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(54) Title: SCREENING ASSAYS FOR COMPOUNDS (57) Abstract The invention is directed to rapid and quantitative assay systems for screening test compounds for their ability to modulate tyrosine kinase or phosphotyrosine phosphatase activities involved in signal transduction. These assays may be practiced in a whole cell or cell-free system. The assays can be used to identify compounds for use in therapeutic applications to disease processes in which tyrosine kinase or phosphatase activity in a signal transduction pathway contributes to a pathological process.		

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SCREENING ASSAYS FOR COMPOUNDS

1. INTRODUCTION

5 The present application is a continuation-in-part of
copen ding application Serial No. 08/279,321, filed July 22,
1994. The present invention relates to rapid, quantitative,
specific, high through-put assay systems for screening test
compounds for their ability to modulate tyrosine kinase or
10 phosphotyrosine phosphatase activity within the cell or in a
cell-free system.

2. BACKGROUND OF THE INVENTION

Protein phosphorylation is a common regulatory mechanism
used by cells to selectively modify proteins carrying
15 regulatory signals from outside the cell to the nucleus. The
proteins that execute these biochemical modifications are a
group of enzymes known as protein kinases. They may further be
defined by the amino acid that they target for
phosphorylation. One group of protein kinases are the
20 tyrosine kinases (TKs) which selectively phosphorylate a
target protein on its tyrosine residues. Some tyrosine
kinases are transmembrane receptors (RTKs), and, upon
activation by a ligand, can autophosphorylate as well as
modify substrates. The initiation of sequential
25 phosphorylation by ligand stimulation is a paradigm that
underlies the action of such effectors as, for example,
epidermal growth factor (EGF), insulin, platelet-derived
growth factor (PDGF), and fibroblast growth factor (FGF). The
receptors for these ligands are tyrosine kinases and provide
30 the interface between the binding of a ligand (hormone, growth
factor) to a target cell and the transmission of a signal into
the cell by the activation of one or more biochemical
pathways. Ligand binding to a receptor tyrosine kinase
activates its intrinsic enzymatic activity. (Ullrich and
35 Schlessinger, Cell 61:203-212, 1990). Tyrosine kinases can

also be cytoplasmic, non-receptor-type enzymes and act as a downstream component of a signal transduction pathway.

Cells express a class of enzymes that complement protein kinases, known as protein phosphatases, which remove the added phosphates. Phosphotyrosine phosphatases (PTPs), specifically remove the phosphates from tyrosine residues of modified proteins, although some may also have enzymatic activity for other phosphoamino acids. As with the TKs, these phosphatases may be transmembrane molecules or they may be localized in intracellular compartments (Walton et al., 1993, Ann. Rev. Biochem. 62:101-120). These proteins also play an integral role in the signal transduction pathways of the cell.

The secondary signal transduction molecules activated by activated receptors result in a signal cascade that regulates cell functions such as cell division, differentiation and survival. These molecules can be cytoplasmic enzymes such as, for example, kinases and phosphatases or can be non-catalytic adapter molecules such as, for example, the Grbs (Growth factor Receptor Bound) (Skolnik, et al., 1991, Cell 65:83-90). Adapter proteins have in common one or two copies of an approximately 100 amino acid long motif called an SH2 (Src homology 2) domain due to its similarity to a motif originally identified in the c-Src cytoplasmic TKs. SH2-containing polypeptides may otherwise be structurally and functionally distinct from one another (Koch, C.A. et al. 1991, Science 252:66-674). SH2 domains directly recognize specific phosphorylated tyrosine residues. There is evidence that the amino acid sequences flanking the phosphorylated tyrosine confers a certain specificity such that a particular SH2 domain will bind preferentially to particular sequences (Koch, C.A., et al. 1991, Science 252:668-674; Cantley, L.C., et al. 1991, Cell 64:281-302).

In addition to SH2 domains, many of the adapter proteins involved in signal transduction contain a conserved motif of 50-75 amino acids known as an SH3 (Src-homology 3) domain (see for example, Schlessinger & Ullrich, 1992, Neuron 9:383-391). In the case of SH3 domains, it is known that proteins with SH3

domains bind to proteins with proline rich regions, such as, for example, -PPPLPP-. A third domain known as the pleckstrin-homology or PH domain, has also been identified in many adapter type proteins (Musacchio et al., 1993, TIBS 5 18:342-348). Much less is known about the biological role of these domains; however, it is believed that they are involved in protein-protein interactions between components of signal transduction pathways (Pawson & Gish, 1992, Cell 71:359-362; Pawsom & Schlessinger, 1993, Current Biology 3:424-442).

10 Reviews describing intracellular signal transduction include Aaronson, S.A., 1991, Science 254:1146-1153; Schlessinger, J., 1988, Trends Biochem. Sci. 13:443-447; and Ullrich & Schlessinger, 1990, Cell 61:203-212.

The profound cellular effects mediated by tyrosine 15 kinases and phosphotyrosine phosphatases have made them attractive targets for the development of new therapeutic molecules. It is known, for example, that the overexpression of tyrosine kinases, such as HER2, can play a decisive role in the development of cancer (Slamon, D.J., et al., 1987, 20 Science, 235:177-182) and that antibodies capable of blocking the activity of this enzyme can abrogate tumor growth. (Drebin, et al. 1988, Oncogene 2:387-394). Blocking the signal transduction capability of tyrosine kinases such as Flk-1 and the PDGF receptor have been shown to block tumor 25 growth in animal models (Millauer, B., et al. 1994, Nature 367:577; Ueno, H., et al. 1991, Science 252:844-848).

Assays currently used for screening drugs/ligands that act on cells containing TKs, in general, involve exposing cells that express the TK to a test substance and either: (a) 30 scoring phenotypic changes in the cell culture as compared to control cells that were not exposed to the test substance; or (b) biochemically analyzing cell lysates to assess the level and/or identity of tyrosine phosphorylated proteins.

This latter approach is illustrated by several 35 methodologies. A common technique involves incubating cells with ligand and radiolabeled phosphate, lysing the cells, separating cellular protein components of the lysate using an

SDS-polyacrylamide gel (SDS-PAGE) technique, in either one or two dimensions, and detecting the presence of phosphorylated proteins by exposing X-ray film. In a similar technique, the phosphorylated proteins are detected by immunoblotting techniques, in which case the phosphate that is detected is not radiolabeled. Instead, the cellular components separated by SDS-PAGE are transferred to a nitrocellulose membrane, where the presence of phosphorylated tyrosines is detected using an antiphosphotyrosine antibody (anti-PY). The anti-PY can be detected by labeling it with a radioactive substance, which then requires scanning the labeled nitrocellulose with a piece of specialized equipment designed to detect radioactivity or exposure of X-ray film. Alternatively, the anti-PY can be conjugated with an enzyme, such as horseradish peroxidase, and detected by subsequent addition of a colorimetric substrate for the enzyme. A further alternative involves detecting the anti-PY by reacting with a second antibody which recognizes the anti-PY, this second antibody being labeled with either a radioactive moiety or an enzyme as previously described. Examples of these and similar techniques are described in Hansen et al., 1993, Electrophoresis 14:112-126; Campbell et al. 1993, J. Biol. Chem. 268:7427-7434; Donato et al., 1992, Cell Growth and Diff. 3:258-268; and Katagiri et al., 1993, J. Immunol. 150:585-593. These techniques have a number of drawbacks that make them unsuitable for rapid screening of a large number of test substances including the undesirable use of radioactive substances, the lack of specificity for particular cellular components of interest due to the use of total cellular lysate, the relatively large amount of handling required to process each sample, and the limited number of samples that can be accommodated by gel electrophoresis.

ELISA-type assays in microtitre plates have been used to test purified substrates. See for example Peraldi et al., 1992, J. Biochem. 285: 71-78; Schraag et al., 1993, Analytical Biochemistry 211:233-239; Cleavland, 1990, Analytical Biochemistry 190:249-253; Farley, 1992, Analytical

Biochemistry 203:151-157; and Lczaro, 1991, Analytical Biochemistry 192:257-261. However, these techniques involve assaying purified components, and thus are unsuitable for evaluating the effects of a test substance on phosphorylation within the normal cellular context. Purified components may be difficult or expensive to obtain in sufficient quantities or may require significant experimental work to develop a purification process for the desired component.

Although variations on these techniques have been described, to the Applicants' knowledge, a rapid screening assay which is highly specific, quantifiable and amenable to testing a large number of test substances has not been developed.

3. SUMMARY OF THE INVENTION

The present invention relates to rapid, quantitative, specific, high through-put assays for screening test compounds such as drugs, ligands (natural or synthetic), ligand antagonists, peptides, or small organic molecules, for their ability to modulate tyrosine kinase or phosphotyrosine phosphatase activities involved in signal transduction.

In accordance with one embodiment of the invention, a target cell that expresses a particular substrate that is phosphorylated or dephosphorylated on tyrosine residues during signal transduction is used. In some cases, the tyrosine kinase is capable of phosphorylating its own specific tyrosine residues, in which case the kinase enzyme is also the substrate. The target cell is exposed to a test substance, and thereafter lysed to release cellular contents, including the protein substrate. The substrate is isolated by contacting the cell lysate with a substrate-specific antibody immobilized directly or indirectly onto a solid support and subsequently washing away the majority of other cellular components, some of which may also be phosphorylated. An assay is performed on the isolated substrate to detect the presence or absence of phosphotyrosine residues on the

substrate compared to lysates of control target cells which were not exposed to the test substance.

Where mimetics of the natural ligand of a signal transducing receptor are to be screened, the target cells are exposed to the test substance in the absence of ligand and the results compared to negative controls that were not exposed to either the ligand or the test substance, and to positive controls that were exposed to ligand only. When inhibitors or enhancers of ligand activity are to be screened, the target cells are exposed to the test compound in the presence of the ligand and results are compared to those of controls where the cells are exposed only to ligand.

In accordance with another embodiment of the invention, the effect of test substances on the ability of the tyrosine kinases and phosphotyrosine phosphatases to phosphorylate and dephosphorylate, respectively, tyrosine residues of a particular substrate can be tested in a cell free system. To this end, an appropriate reaction mixture is prepared containing the substrate and kinase (these are one and the same in the case when autophosphorylation is measured) or the phosphorylated substrate and phosphatase, which can also be one and the same. The kinase reaction is initiated in the presence of ATP and divalent cations. An immunoassay is performed on the reaction product to detect the presence or absence of phosphotyrosine residues on the substrate, and results are compared to those obtained for controls.

Detection of the presence or absence of phosphotyrosine residues on a target substrate is performed in one of several ways. One method (immunoassay or sandwich ELISA) utilizes a signal-generating anti-phosphotyrosine antibody to detect phosphotyrosine residues on the immobilized substrate thereby measuring the degree of phosphorylation of the substrate. In a second method, detection of the presence or absence of phosphotyrosine is accomplished by the binding of a specific SH2 domain or SH2 domain containing protein to the immobilized substrate. Said SH2 domain or SH2 domain containing protein will be relatively specific for a particular phosphorylated

tyrosine. SH2 domain containing proteins are, for example but not limited to, Grb molecules such as Grb2 (Margolis, B., et al. 1989, Cell 51:1101-1107; Bjoige, et al. 1990, Proc. Nat. Acad. Sci. USA 87:3816-3820; Pelicci, et al. 1992, Cell 5 70:93-104; Fu & Zhang, 1993, Cell 74:1135-1145). The degree of binding of the SH2 domain or SH2 domain containing protein can be accomplished with a signal-generating anti-SH2 domain or anti-SH2 domain containing protein antibody or by, for example, using an SH2 domain or SH2 domain containing protein 10 fused to a signal-generating protein, such as, for example GST (glutathione-s-transferase, Tsang, V.C.W., et al. Methods in Enzymology 92:377, 1983).

The invention is demonstrated by way of working examples using a whole cell assay to screen for inhibitors of kinase 15 activities of EGF-receptor and HER-2, and inhibitors of phosphatase activity that dephosphorylates insulin receptor (Section 6, infra); a cell-free assay to screen for inhibitors of the EGF-receptor (Section 7, infra), and a cell-free assay to screen for inhibitors of phosphatase 1B (Section 8, infra).

20

4. DEFINITIONS

The following terms whether used in the singular or plural shall have the meanings indicated:

Target Cell: a cell which expresses a tyrosine kinase or 25 phosphotyrosine phosphatase of interest, and which further contains a substrate which can be phosphorylated or dephosphorylated as a result of signal transduction. The tyrosine kinase or phosphatase may be naturally expressed by the target cell or engineered into the target cell using 30 recombinant DNA techniques well known in the art.

Test Substance: a chemically defined compound or mixture of compounds (as in the case of a natural extract or tissue culture supernatant) whose effect on the phosphorylation of/by the tyrosine kinases or the dephosphorylation by the tyrosine 35 phosphatases of a target cell is determined by the assay of the invention.

Substrate: a protein which is acted on by tyrosine kinase or tyrosine phosphatases such that it is either phosphorylated or dephosphorylated on tyrosine residues. Tyrosine kinases and phosphatases can act as both an enzyme
5 and a substrate.

Anchoring Molecule: an antibody or other protein which binds specifically with high affinity to a substrate of a tyrosine kinase or phosphatase and which is immobilized onto a solid phase. The anchoring molecule can be bound directly or
10 indirectly to the solid phase. Indirect binding can be accomplished, for example, by first coating the solid phase with an antibody which binds specifically to the anchoring molecule and subsequently adding the anchoring molecule.

Detection Molecule: an antibody or other protein or
15 amino acid sequence that specifically binds to phosphorylated tyrosine residues, or to a particular phosphorylated tyrosine residue, and which is used in the assay to detect phosphorylated tyrosines on the substrate bound by the anchoring molecule. The detection molecule may be used as an
20 anchoring molecule, in which case the anchoring molecule as defined above can be used to detect the level of bound substrate. An example of a protein other than an antibody that specifically binds to phosphorylated tyrosines is a SH2 domain or a protein containing an SH2 domain.

25

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for screening compounds that modulate tyrosine kinase or phosphotyrosine phosphatase activities involved in signal transduction. The
30 assays of the invention involve monitoring the phosphorylation or dephosphorylation of tyrosine residues on selected substrates involved in signal transduction in a target cell and can be practiced in a whole cell or a cell-free system.

Test substances which mimic, enhance or inhibit the
35 signal transduction which is the result of ligand binding to the receptor may be readily identified using the assays of the invention. The test substances include but are not limited to

hormones that interact with membrane-bound tyrosine kinase receptors, or may be drugs that exert effects on the target cells through the modulation of tyrosine kinases or phosphatases. In this regard, the present invention provides a
5 method for determining the effect of a test substance on specific tyrosine kinases or phosphatases or their substrates in a target cell.

In one embodiment of the invention, target cells that express a protein substrate that is phosphorylated or
10 dephosphorylated on a tyrosine residue during signal transduction are exposed to a test substance and, thereafter, lysed to release cellular contents, including the substrate of interest. Where mimics of the natural ligand for a signal transducing receptor are to be screened, the target cells are
15 exposed to the test substance and compared to positive controls which are exposed only to the natural ligand and to negative controls which were not exposed to either the test substance or the ligand. Where inhibitors or enhancers of ligand-induced signal transduction are to be screened, the
20 target cells are exposed to the natural ligand in the presence of the test substance and compared to controls which are not exposed to the test substance. The substrate of interest is isolated by incubating the cell lysate with a substrate-specific anchoring molecule bound to a solid support and
25 thereafter washing away non-bound cellular components. A detection procedure is performed to assess the presence or absence of phosphotyrosine residues on the substrate as compared to lysates of control cells which were not exposed to the test substance.

30 This assay offers several advantages. The exposure of the test substance to a whole cell allows for the evaluation of its activity in the natural context in which inhibitors or enhancers of tyrosine kinases and phosphotyrosine phosphatases may act. As this assay is performed in whole cells and is
35 based on the use of a known phosphorylated substrate, it does not require redesign or the use of reagents specific for the particular kinase or phosphatase responsible for

phosphorylating or dephosphorylating said substrate. In addition, radioactive labeling of the target cell proteins is not required in the assay. The assay can detect the effects of the test compounds on cellular kinase or phosphatase activities even if the direct target of the compound is unknown. Because this assay, and those described below, can readily be performed in a microtiter plate format, the assays described can be performed by an automated robotic system, allowing for testing of large numbers of test samples within a reasonably short time frame.

The anchoring molecule should be highly specific for the substrate of interest. It is preferred that the anchoring molecule be an antibody, hereinafter referred to as an anchoring antibody. The anchoring antibody is preferably generated against a unique epitope of the substrate, most preferably an epitope wherein binding by the anchoring antibody will not interfere with binding of the detection antibody or SH2 domain or ligand binding. The anchoring antibody may be a monoclonal antibody but can also be a polyclonal antibody.

The detection procedure used to assess the phosphorylation state of the substrate may employ an anti-phosphotyrosine antibody or a peptide that recognizes and binds to phosphorylated tyrosines. Said amino acid sequence may be, for example, an SH2 domain and may furthermore be an isolated SH2 domain or an intact protein containing an SH2 domain. The detection antibody is preferably a polyclonal antibody to maximize the signal but may also be specific monoclonal antibodies which have been optimized for signal generation.

An alternate embodiment of the invention relates to methods for determining the effect of test compounds on the ability of tyrosine kinases to autophosphorylate or phosphorylate the substrate of interest in a cell-free system, or the ability of tyrosine phosphatases to dephosphorylate such substrates in a cell-free system. To assess modulation of enzyme activity, the test substance is added to a reaction

mixture containing the kinase or phosphatase and its substrate bound to a solid support by an anchoring antibody. The kinase reaction is initiated by the addition of ATP. A detection procedure as described above is performed on the substrate to
5 assess the presence or absence of the phosphorylated tyrosine residues, and results are compared to those obtained for controls i.e., reaction mixtures to which the test substance was not added. A similar procedure is used to assess autodephosphorylation by phosphotyrosine phosphatases.

10 A further aspect of this embodiment of the invention allows the user to distinguish test substances that inhibit the interaction between the kinase and its substrate from test substances that inhibit the interaction between the kinase and ATP. Test substances that inhibit the interaction between the
15 kinase and its substrate may be more target specific than those that inhibit the interaction between the kinase and ATP.

The assays of the invention can be used as a primary screen to assess the activity of a previously untested compound or extract, in which case a single concentration is
20 tested and compared to controls. This assay can also be used to assess the relative potency of a compound by testing a range of concentrations, in a range of 100 μ M to 1 pM, for example, and computing the concentration at which the amount of phosphorylation is reduced by one-half (IC50) compared to
25 controls in the case of inhibitors or increased by one-half relative to the natural ligand in the case of enhancers.

~~These assays can be used to identify compounds which~~
modulate protein kinase or protein phosphatase activity. It is further contemplated that the assays of the invention can
30 be used to diagnose clinical conditions in which the effect of a known compound on protein kinases or protein phosphatases is altered by reference to a suitable control experiment.

The identification of compounds that modulate protein kinase or protein phosphatase activity can have utility in the
35 selection of compounds to treat neoplastic disorders in which cell proliferation has been shown to correlate with an increase or decrease in kinase or phosphatase activity. For

example, in a case where overexpression of a protein kinase, such as HER2, has been shown to correlate with the aberrant growth characteristics of a cell line or tumor, the identification of a compound that can inhibit protein kinase
5 activity may restore normal growth patterns and reverse oncogenicity. In a tumor cell line or tumor in which protein phosphatase activity is decreased, for example, the identification of a compound that stimulates protein phosphatase activity may restore normal growth and reverse
10 oncogenicity.

The compounds identified in the assays of the invention may be used as therapeutic agents against any diseases in which modulation of tyrosine kinase or tyrosine phosphatase activity reverses a pathological process in cells, tissues or
15 organs.

In a further embodiment of the invention, the assays described herein can be used to determine if a cell line or tumor in which aberrant kinase or phosphatase activity contributes to a pathological process or disease state will
20 respond to a compound that modulates kinase or phosphatase activity and therefore reverse the pathological process or disease state. In this manner, therapeutic regimens may be devised using the assays of the invention on cells derived from patients, avoiding the necessity to subject the patient
25 to trial and error.

In addition, if a compound known to modulate kinase or phosphatase activity is shown to cause phenotypic changes in a target cell, these assays may be used to identify the substrates involved in the signal transduction pathway
30 responsible for the phenotype. Furthermore, the specific kinases or phosphatases operating in the signal pathway that causes a particular phenotype may be identified through an analysis of these substrates.

The whole cell assay of the invention described herein
35 can be performed, for example, by utilizing pre-packaged kits comprising any or all of the reagents of the assay, such as a solid phase coated with an anchoring molecule to a substrate

of interest, or a detection molecule. The cell-free assays of the invention may be performed, for example, by utilizing pre-packaged kits comprising any or all of the reagents of the assay, such as an enzyme, substrate, anchoring molecule, a
5 solid phase coated with an anchoring molecule to a substrate of interest, or a detection molecule.

The invention is illustrated in greater detail in the sections that follow and demonstrated by the working examples infra.

10

5.1. SELECTION OF ASSAY PARAMETERS

Practice of the whole cell assay of the invention requires the selection of an anchoring antibody (or molecule) and a target cell. The anchoring antibody should have a high
15 binding affinity for and be highly specific for the target substrate. The anchoring antibody may be a monoclonal antibody or a polyclonal antibody (see below). Monoclonal antibodies or polyclonal antibodies selective for the substrate are selected by techniques well known in the art.
20 Immunoblots can be performed using lysates from cells that express the target substrate to determine specificity. The preferred antibody will only detect the substrate, preferably where greater than 100,000 molecules per cell. An alternative method for determining specificity is immunoprecipitation.
25 The binding affinity of the monoclonal antibody or polyclonal antibody for the substrate can be determined by the relative strength of the signal generated in the immunoblot or by other techniques well known in the art.

To select a target cell a known number of cells
30 expressing the substrate is lysed and serial dilutions of the lysate are applied to wells in a 96 well microtiter plate that have been precoated with the anchoring antibody. After allowing the substrate to bind to the antibody, the unbound material is washed away and the amount of bound substrate is
35 determined using known immunoassay techniques. In order to have the proper signal to noise ratio one must be able to detect the target molecule in approximately 1×10^4 cell

equivalents per well. The maximum number of cells allowable per well is generally $<1 \times 10^5$ due to space constraints although this number may be somewhat larger or smaller depending on the cell type.

- 5 The preferred amount of anchoring antibody and the preferred number of cells to be seeded in each well can be determined empirically by varying the amount of anchoring antibody used to coat the well and the amount of cell lysate (whole cell equivalents) added per well. The ideal
10 concentration of these two components will produce a signal which will reflect a specific phosphotyrosine content in a linear part of the curve.

5.2. PREPARATION OF ANTIBODIES USED IN THE ASSAYS

- 15 Various procedures known in the art may be used for the production of antibodies to the cellular substrates of interest and/or to the phosphotyrosine residues. Monoclonal or polyclonal antibodies specific for the substrate of interest which may advantageously be used as anchoring
20 antibodies should be prepared against unique epitopes of the substrate to minimize cross reactions with other substrates. Similarly, the detection antibody should only react with a phosphorylated tyrosine amino acid. Monoclonal antibodies or polyclonal antibodies with high binding affinities for such
25 unique epitopes may be used, preferably those that will not interfere with ligand binding if the substrate is also a receptor. For example, where the substrate is a receptor involved in signal transduction, the extracellular domain, which bears unique epitopes that may be involved in ligand
30 recognition and binding, may advantageously be used as the immunogen. For non-receptor substrates where the amino acid sequence is known, non-conserved, variable regions of the protein may be used as immunogens. Anti-phosphotyrosine antibodies may be generated using phosphotyramine epitopes as
35 described in the examples infra. (Fendly et al., 1990, Cancer Research 50:1550-1558)

The antibodies used in the immunoassay include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and an Fab expression library. For the production of antibodies, various host animals, including but
5 not limited to rabbits, mice, rats, etc., may be immunized by injection with the particular antigen in a suitable adjuvant or by injecting the epitope conjugated to an immunogenic carrier. Preferably conjugates are prepared so that the most unique epitope of the substrate will be most accessible to
10 antibody. When the substrate of interest is a cell-surface receptor, whole cells expressing said receptor may be used as the antigen. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and
15 incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and
20 *Corynebacterium parvum*.

Monoclonal antibodies may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally
25 described by Koehler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Human antibodies may
30 be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. 80:2026-2030) or by transforming human B cells with EBV virus in vitro (Cole et al., 1985, in, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). Techniques developed for the production
35 of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by

splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

Techniques for the production of single chain antibodies (U.S. Patent 4,946,778) can also be used to produce substrate-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to antigens.

Antibody fragments which contain binding sites specific for the substrate may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments.

The antibodies may be stored and purified using methods which are well known to those skilled in the art (e.g., see "Antibodies, A Laboratory Manual, eds. Harlow & Lane, Cold Spring Harbor Laboratory, 1988, Ch. 8).

Alternatively, polyclonal or monoclonal antibody specific for the substrate of interest may be obtained from commercial sources.

5.3. PREPARATION OF PHOSPHOTYROSINE SPECIFIC PROTEINS USEFUL IN THE ASSAYS

The phosphotyrosine specific proteins or peptides used for the detection procedures are produced by methods well known to those skilled in the art. They may be purified from cells that natively express the protein, or may be produced by recombinant means and purified from genetically engineered cells. A variety of host-expression vector systems may be

utilized to express the coding sequences of said protein or peptides. These include but are not limited to microorganisms such as bacteria (e.g., E.coli, B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA
5 expression vectors containing PTK or adaptor protein coding sequences; yeast (e.g., Saccharomyces, Pichia) transformed with recombinant yeast expression vectors containing the protein or peptide coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g.,
10 baculovirus) containing the protein or peptide coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the
15 protein or peptide coding sequence; or mammalian cell systems (e.g. COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter;
20 the vaccinia virus 7.5K promoter).

In bacterial systems expression vectors include but are not limited to the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the protein or peptide coding sequence may be ligated individually into the vector in
25 frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with
30 glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free reduced glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease
35 cleavage sites so that the cloned protein or peptide can be released from the GST moiety. Alternatively, the fusion protein can be left intact. In the case where the

non-phosphotyrosine binding portion of the fusion protein is an enzyme, said fusion protein can be reacted with a colorimetric substrate as described below. In another alternative, the non-phosphotyrosine binding portion of the fusion protein can remain uncleaved so that it may be detected by an antibody specific to it.

In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The protein or peptide coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is expressed. (E.g., see Smith et al., 1983, J. Virol. 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the protein or peptide coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing proteins or peptides in infected hosts (e.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an protein gene, including its own initiation

codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the coding sequence is inserted, exogenous translational control
5 signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of
10 a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

15 The proteins or peptides may be produced synthetically. See, for example, Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman and Co., NY, which is incorporated herein, by reference, in its entirety.

20 5.4. IMMOBILIZED PHASE

Solid phases used for the immobilization of a substrate may be prepared by coating with the anchoring molecule specific for the substrate. The anchoring molecule may be directly immobilized onto the solid phase, or, alternatively,
25 may be indirectly immobilized to the solid phase by an antibody bound to the solid phase which is specific for the anchoring molecule. In the case where a polyclonal antibody is used as the anchoring molecule, the solid phase may first be coated with an anti-Ig that binds to the polyclonal
30 antibody and indirectly immobilizes it to the solid phase.

The solid phase may comprise a microtiter plate, a stick, tube, disc, fiber or the like, or a microtiter plate. A preferred solid phase is a 96 well microtiter plate such as those available from Corning, Cynatech and Nunc. Particularly
35 preferred 96 well plates are the Corning, Nunc MaxiSop and Dynatech Immulon I and IV. Ideal conditions for maximum coating with the anchoring antibody can vary with pH, ionic

strength and antibody concentration. Preferred conditions will be pH 6 - 9.5, 0 - 200 mM NaCl and 1 - 10 μ g/ml of antibody. Generally 150 μ l per well is used. The anchoring molecule may be attached to the solid phase by any of a
5 variety of methods known to those skilled in the art, including but not limited to non-covalent and covalent attachments. For example, the anchoring molecule can be applied to the solid phase in buffer for a specified period of time to allow the anchoring molecule to absorb to the solid
10 phases, at which time the buffer is removed and blocking buffer is added. Blocking buffer may be dry milk, gelatin, bovine serum albumin or ethanolamine as a 1 - 5 % solution in a neutral pH Tris-HCl buffer. Following the removal of the blocking buffer, the solid phase is washed and ready for use.
15 In the case of indirect immobilization, the substrate specific antibody is added after blocking.

The anchoring molecule and the coated solid phase may be prepared ahead of time and stored until required for use in the assay.

20

5.5. SIGNAL-GENERATING DETECTION SYSTEMS

The detection molecules are modified by the addition of a signal-generating system to allow for detection of the detection molecules bound to phosphotyrosines in the assay
25 system. The detection molecules may be labeled directly or can be detected using a secondary reagent that will detect the detection molecule. Such signal generating systems include, but are not limited to, enzyme-linked systems (such as horseradish peroxidase or alkaline phosphatase), radiolabels,
30 fluorescent labels, light-emitting labels, light-absorbing labels, or dyes (e.g., See "Antibodies, A Laboratory Manual, eds. Harlow & Lane, Cold Spring Harbor Laboratory 1988, Ch. 9).

In the case of conjugated enzymes, an appropriate
35 substrate, such as a colorimetric substrate, is added. Specific substrates used for detection include ABTS (horseradish peroxidase), DAB, AEC, BCIP/NT (alkaline

phosphatase) and BCIG (beta-galactosidase). The binding of the enzyme-conjugated anti-IgG can be then detected quantitatively by techniques well known in the art.

In a specific example described herein, an anti-rabbit
5 biotinylated IgG peroxidase conjugate is incubated with the substrate ABTS in order to facilitate detection of the bound antibody. After the reaction is stopped, the product of this reaction can be detected by determination of the O.D. at 410 nm. (Background signal measured at 630 nm is subtracted.)

10

5.6. EXPOSURE OF TARGET CELLS TO TEST
SUBSTANCE AND PREPARATION OF CELL
LYSATE

The target cells used in the present invention may express a tyrosine kinase that itself is a substrate for
15 phosphorylation, or that phosphorylates other protein substrates. These cells may have a tyrosine kinase that is natively expressed or they may be genetically engineered to express a specific tyrosine kinase. The kinase may be a receptor tyrosine kinase that is responsive to an exogenous
20 ligand, or the tyrosine kinase may be capable of self-phosphorylation even in the absence of ligand binding. The tyrosine kinase may be a cellular kinase that is not membrane-bound, but is activated in a signal transduction pathway. The target cells of the present invention may also
25 natively express a phosphotyrosine phosphatase (PTP), or they may be genetically engineered to express a PTP. The PTP may be a receptor phosphotyrosine phosphatase or a cytoplasmic enzyme. These target cells may have a PTP that is constitutively active in regulating the level of
30 phosphorylation on substrate proteins that are capable of self-phosphorylation or are phosphorylated by a basal kinase activity, in the absence of ligand binding.

The test substances can be any of a variety of substances including but not limited to hormones that interact with a
35 membrane-bound tyrosine kinase receptor, or drugs that exert their effect on the target cells through the modulation of

intracellular tyrosine kinases or phosphatases. The test compounds may exert their effect through interference with ligand induced activation of a membrane-bound receptor. The test substances may be molecules that are the physiologic
5 ligands for the receptor. They may be molecules that are not the physiologic ligands for the receptor and can be tested for an effect on the enzymatic activity of a specific receptor tyrosine kinase or phosphatase. The compounds may be drugs that modulate a receptor tyrosine kinase or phosphatase in the
10 absence of ligand. They may be drugs that inhibit the ligand-dependent activation of a receptor tyrosine kinase or phosphatase. The present invention provides a method for the determination of the effect of a drug on specific tyrosine kinases or phosphatases by analysis of their substrates
15 obtained from the target cell.

The target cells are grown using standard protocols for tissue culture maintenance, in which the cells are fed with appropriate medium, and incubated at the appropriate temperature with CO₂, if required. The substance to be tested
20 is added to the growth medium, and the conditions of incubation with the cells are dictated by the particular assay. If a test substance is to be tested for its effect on ligand-dependent modulation of a receptor, the ligand which activates the receptor is added to the target cell in the
25 presence of the test compound.

Following all exposures to test compounds and/or ligands, a cell lysate is prepared for analysis by immunoassay of the phosphorylated substrate. Cell lysates may be prepared by known techniques in the art in which the cell membrane is
30 solubilized by the addition of a detergent, and the intracellular contents stabilized with the addition of buffers, protease inhibitors and phosphatase inhibitors. Detergents include but are not limited to Triton X-100, Tween 20, NP-40, or SDS. Protease inhibitors include but are not
35 limited to PMSF, leupeptin, EDTA and aprotinin. Phosphatase inhibitors include but are not limited to sodium orthovanadate sodium pyrophosphate and EDTA.

5.7. EXPOSURE OF SUBSTRATE TO TEST
SUBSTANCE IN A REACTION MIXTURE

In an alternative embodiment, the effect of the test substances on the phosphorylation of the substrate of interest can be assessed in a cell-free reaction mixture. For example, to determine the effect of a test substance on kinase activity, the test substance can be incubated with the substrate of interest in a reaction mixture containing the kinase (where autophosphorylation is to be assessed, the substrate and the kinase are one and the same). The kinase reaction is then initiated by the addition of ATP and appropriate cations. The substrate is then immunoassayed for the presence of phosphotyrosine residues using an anti-phosphotyrosine detection antibody or a phosphotyrosine binding protein or peptide such as one containing an SH2 domain. The effect of the test substance on the kinase activity is reflected by the degree of phosphorylation detected in the samples treated with the test compound as compared to untreated controls.

The cell-free approach can likewise be used to determine the effects of the test substance on phosphatase activity. To this end the test compound is first incubated with a phosphorylated substrate of interest, which can be the PTP itself, in a reaction mixture to which the phosphatase is added. The substrate is then immunoassayed for the presence of phosphotyrosine residues using an antiphosphotyrosine detection antibody or a phosphotyrosine binding protein or peptide such as one containing an SH2 domain. The effect of the test substance on the phosphatase activity is reflected by the degree of dephosphorylation detected in the samples treated with the test substance as compared to untreated controls. The phosphorylated substrate used in the assay can be obtained from cell lysates of target cells which were activated by their natural ligand. Alternatively, the substrate can be obtained from unactivated cell lysates and phosphorylated in vitro in a reaction mixture containing the

kinase (where autophosphorylation is involved, the kinase and substrate of interest are one and the same), ATP and appropriate cations.

While the reaction product can be detected by any of the
5 immunoassays described herein, a particularly rapid approach is described in the working examples, infra. Briefly, the substrate of interest can be immobilized using an anchoring antibody prior to conducting the kinase and/or phosphatase reactions in the presence of the test substances, so that all
10 reactions are carried out on the solid phase.

6. EXAMPLE: WHOLE CELL SCREENING ASSAYS

The whole cell assays assess the potential of an exogenous test substance, applied to a target cell expressing
15 a tyrosine kinase or tyrosine phosphatase, to modulate the activity of that enzyme. In these two examples, conditions are described for assays assessing the ability of test compounds to inhibit the kinase activity of EGFR or HER2, and to inhibit the phosphatase activity that dephosphorylate the
20 phosphotyrosine residues on insulin receptor (IR). Those skilled in the art will recognize that such conditions can be used with other targets, such as Platelet-Derived Growth Factor receptor (PDGFR), by using a different target cell and anchoring antibody. In the case of EGFR, exogenous ligand is
25 added to stimulate the kinase activity of the receptor. In the case of HER2, kinase activity is stimulated without the addition of exogenous ligand. In the case of IR, no insulin is added because the receptor is capable of self-phosphorylation even in the absence of ligand binding. After
30 exposure to the test substances, cell lysates were prepared and added to microtiter plates coated with anti-target antibody. Phosphorylation of the immobilized target was detected using anti-phosphotyrosine antisera.

35

6.1. KINASE INHIBITOR SCREENING ASSAY

6.1.1. MATERIALS AND REAGENTS

1. The cell line used for the EGFR assay was NIH3T3
5 clone 7 (EGFR/c7, Honegger, et al. Cell 51:199-209, 1987)
engineered to over-express human EGFR. Growth media for these
cells is DMEM (Gibco) containing 10% calf serum 1% L-glutamine
and 20 mM Hepes. The cell line used for the HER2 assay was BT
474 (ATCC HTB20) which over-expresses HER2. Growth media for
10 these cells is RPMI (Gibco) plus 10% fetal calf serum plus
GMS-G (Gibco supplement) plus glutamine.
2. The anchoring antibody used for the EGFR assay was a
monoclonal antibody that recognizes the EGFR extracellular
domain purchased from UBI (catalogue No. 05-101). An antibody
15 recognizing the extracellular domain of HER2 was prepared,
using techniques well know in the art, by immunizing mice with
NIH3T3 cells engineered to over-express HER2. Hybridoma
supernatants were screened for binding to HER2 expressing
whole cells, and one clone (SUM02) was selected and grown in
20 DMEM (GIBCO) + 1% calf serum. The antibody was purified by
Protein A agarose chromatography using a citric acid elution
buffer, after which it was immediately neutralized. The
antibody solution was dialyzed against PBS coating buffer (see
below) and stored at -80 C until used.
- 25 3. EGF was purchase from Toyobo, Co. Ltd., Japan (EGF
201), and kept as a stock solution of 16.5 μ M.
4. Rabbit polyclonal antiphosphotyrosine antibody
(anti-PY) was prepared according to Fendly, et al., 1990,
Cancer Research 50:1550-1558.
- 30 5. Goat anti-rabbit IgG horseradish peroxidase
conjugate (Tago, Burlingame, CA, Cat.No. 4520 or Biosource
Int., Camarillo, CA) was used as the detection antibody.
6. TBST buffer: 50 mM Tris-HCl (pH 7.2), 150 mM NaCl,
0.1% Triton X-100.
- 35 7. Blocking buffer: TBST plus 5% milk (Carnation
instant non-fat dry milk)

8. 5X HNTG buffer : 100 mM HEPES, 750 mM NaCl, 50% glycerol, 1% Triton X-100, pH 7.2

9. ABTS solution: 100 mM citric acid, 250 mM Na₂HPO₄, 0.5 mg/ml ABTS (2,2'-azinobis(3-ethylbenzthiazlinesulfonic acid), pH 4.0.

10. Cell lysis buffer: For 10 mls - 2 ml 5X HNTG, 0.1 ml of 0.5 M EDTA-HCl (pH 7.0), 0.1 ml of 0.5 M Na₃VO₄ (kept as a 100X stock at 80 C in aliquots), 1 ml of 0.2 M NaP₂O₇, and 7.3 mls distilled water.

11. Hydrogen peroxide: 30% solution.

12. Test substances were prepared according to Ohmichi, et al., 1993, Biochemistry 32(17):4650-4658; and Gazit, A., et al., 1991, J. Med. Chem. 34(6):1896-1907.

6.1.2. PREPARATION OF ASSAY PLATES

Microtiter plates (96 well) were coated with anchoring antibody at 0.5 µg per well in PBS (GIBCO), 150 µl final volume/well, covered with parafilm, and stored overnight at 4°C. Coated plates are good for up to 10 days when stored at 4°C. Before using, the coating buffer was removed and replaced with blocking buffer (200 µl), then incubated, shaking, at room temperature for 30 minutes. Blocking buffer was removed and the plate washed 4 times with TBST buffer.

6.1.3. SEEDING CELLS

Cells were grown in tissue culture dishes until 80-90% confluence then collected by trypsinization (0.25% trypsin-EDTA (Gibco)). The reaction was stopped with the addition of medium containing 10% FCS. The cells were suspended in fresh medium, and centrifuged once at 1500 rpm, room temperature, for 5 minutes. The cells were resuspended in fresh medium and transferred to 96 well tissue culture plates (Corning, 25806-96) (10,000 cells/well for EGFR/c7 starved at 0.5% FCS for 40-48 hrs; 50,000 cells/well for BT474) at 100 µl/well. The plates were incubated at 37°C in 5% CO₂, overnight.

6.1.4. ASSAY PROCEDURE

Media in the wells was replaced by serum free growth medium (DMEM or RPMI), 90 μ l per well. Serial dilutions of test compound stocks (10 mg/ml in DMSO) were diluted 1:10 into growth media (DMEM or RPMI) and 10 μ l added per well for a final concentration range of 100 μ M to 1 nM. (In a primary screen, 10 mg/ml test compound stock (in DMSO) is diluted 1:10 into serum free growth medium for final concentrations of 1:200 for the test substance and 0.5% for DMSO in the well.) Control wells received DMSO and serum free medium only. The cells were incubated for 1 hour at 37 C, 5% CO₂.

For the EGFR assay, EGF was diluted in DMEM such that upon transfer of 10 μ l of dilute EGF (1:12 dilution, a 25 nM concentration is attained in the microtitre well. After the 1 hour incubation with the test drug, 10 μ l of EGF was added per well. Control wells received DMEM alone. The plate was incubated, shaking, at room temperature an additional 5 minutes.

For both assays, after incubation all solution was removed and the wells washed twice with PBS. Cell lysis buffer (100 μ l) was added to each well, then the plate was left on ice for 5-10 minutes. The assay plates were emptied by removing the blocking buffer and washing with TBST.

Cells were scraped from the microtiter wells and homogenized using a pipette tip and repeated aspirating and dispensing. The cell lysate was transferred to the assay plate wells, and the target substrate was allowed to bind for 1 hour at room temperature, shaking. The lysate was removed, and the plate washed 4 times with TBST.

Phosphotyrosine was detected by the addition of anti-PY (100 μ l per well, diluted 1:3,000 with TBST), then incubated, shaking, at room temperature for 30 minutes. The anti-PY solution was removed, and the plate washed 4 times with TBST. Detecting antibody was added (100 μ l per well, diluted 1:3,000 with TBST), and the plate was incubated for 30 minutes at room temperature, shaking. The detecting antibody solution was removed, the plates were washed with TBST (4x) and fresh

ABTS/H₂O₂ was added (100 μ l per well) to start color development. (ABTS/H₂O₂ is prepared with 1.2 μ l H₂O₂ to 10 ml ABTS.) Color was allowed to develop for 20 minutes at room temperature. Color development may be stopped by the optional
5 step of adding 50 μ l 5N H₂SO₄. Optical density is measured at 410 nM (Dynatec MR5000).

6.1.5. RESULTS

Example results are shown in Table I (EGFR) and Table II
10 (HER2).

TABLE I

EFFECT OF TEST SUBSTANCES ON
KINASE ACTIVITY OF EGF-R ACTIVATED BY EGFR

15	Test Substance	IC50 (μ M)
	42	> 50
	957	43.6
	48	> 100

TABLE II

EFFECT OF TEST SUBSTANCES ON HER2

20	Test Substance	IC50 (μ M)
	42	> 50
25	48	41.5
	56	30.1

6.2 PHOSPHATASE INHIBITOR SCREENING ASSAY

30

6.2.1. MATERIALS AND REAGENTS

1. The cell line used for the IR assay was NIH3T3 (ATCC# CRL 1658) engineered to over-express the human IR (H25 cells). Growth media for these cells is DMEM (Gibco) containing 10% fetal bovine serum, 1% L-glutamine and 20 mM
35 Hepes.

2. The anchoring antibody used was BBE which recognizes the extracellular domain of human IR, and was purified by the Enzymology Laboratory, Sugen Inc.
3. PBS (Gibco): KH_2PO_4 0.20 g/l, K_2HPO_4 2.16 g/l, KCl 0.20 g/l, NaCl 8.00 g/l, pH7.2.
4. Rabbit polyclonal antiphosphotyrosine antibody (anti-pTyr) was prepared by the Enzymology Laboratory, Sugen, Inc.
5. Goat anti-rabbit IgG POD conjugate (Tago, Burlingame, CA, Cat.No. 6430) was used as the detection antibody.
6. TBST buffer: 50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, adjusted to pH7.2 with 10N HCl.
7. Blocking buffer: PBS plus 5% milk (Carnation instant non-fat dry milk).
8. 5X HNTG buffer: 100 mM HEPES, 750 mM NaCl, 50% glycerol, 0.5% Triton X-100, pH 7.5.
9. ABTS solution: 100 mM citric acid, 250 mM Na_2HPO_4 , 0.5 mg/ml ABTS (2,2'-azinobis(3-ethylbenzthiazlinesulfonic acid), adjusted to pH 4.0 with 1N HCl.
10. Cell lysis buffer: HNTG containing 1mM Na_3VO_4 (0.5M solution kept as a 100X stock at -80°C in aliquots), 5mM NaP_2O_7 , and 5mM EDTA prepared fresh and keep on ice until ready for use.
11. Hydrogen peroxide: 30% solution.

6.2.2. PREPARATION OF ASSAY PLATES

Microtiter plates (96-well, Easy Wash ELISA plate, Corning 25805-96) were coated with the anchoring antibody BBE at 0.5 μg per well in PBS (GIBCO), 100 μl final volume/well, for at least 2 hours at room temperature before use or overnight at 4°C . Before use, the coating buffer was replaced with 100 μl blocking buffer, and the assay plate was shaken at room temperature for 30 minutes. The wells were washed three times with water and once with TBST buffer before adding lysate.

6.2.3. SEEDING CELLS

Target cells were grown in 15cm culture dish (Corning 25020-100) in DMEM media containing 10% fetal bovine serum (FBS) until 80-90% confluent. The cells were harvested with
5 trypsin-EDTA (0.25%, 0.5ml, Gibco), resuspended in fresh medium containing 10% FBS, 1% L-glutamine and Hepes, and transferred to round bottom 96-well tissue culture plates (Corning 25806-96) at 25,000 cells/well, 100µl/well. The cells were incubated at 37°C at 5% CO₂ for 24 hours. The
10 media was changed by inverting the plate, and adding DMEM medium containing 0.5% FBS and Hepes. The cells were further incubated overnight at 37°C, 5% CO₂.

6.2.4. ASSAY PROCEDURE

15 The assay was set up in the 96-well tissue culture plate. Before adding test substance to the cells, media in the wells was replaced by serum free DMEM medium, 90 µl per well. Positive control wells received 80 µl DMEM. Negative controls received 90µl DMEM. Test substances were diluted 1:10 with
20 DMEM and 10 µl/well of the diluted test substances were transferred to the cells in the wells to achieve a final dilution of 1:100. Positive and negative control wells received 10 µl/well of dimethyl sulphoxide (DMSO) to achieve a final concentration of 1%. Positive control wells
25 additionally received 10 µl/well of 0.1 M Na₃VO₄ so that the final concentration is 10 mM. The microtiter plate was shaken for 1 minute before incubation at 37°C and 5% CO₂. After 90 minutes of incubation, the media was removed by inversion of the plate, and 100µl/well of lysis buffer was added to the
30 cells. The tissue culture plate was shaken for 5 minutes and then placed on ice for 10 minutes. The cells were homogenized by repeated aspirating and dispensing, and the cell lysates were transferred to the corresponding wells of a precoated assay plate.
35 The substrate in the cell lysates was allowed to bind to the anchoring antibody for 1 hour shaking at room temperature. The lysates were then removed, and the assay plate was washed.

All assay plate washings were done by rinsing in water three times followed by one rinse in TBST. The plate was dried by tapping it on paper towels. Phosphotyrosine was detected by adding to the wells 100 μ l/well anti-pTyr antiserum (diluted 1:3000) with TBST and incubating for 30 minutes shaking at room temperature. The unbound excess anti-pTyr antiserum was then removed, and the assay plate was washed. A secondary antibody diluted 1:3000 with TBST, was added to the wells, and incubated for 30 minutes shaking at room temperature. The detection antibody was then removed, the plate washed, and fresh ABTS/H₂O₂ (1.2 μ l 30% H₂O₂ to 10ml 0.5mg/ml 2,2'-azinobis(3-ethylbenzethiazoline)sulfonic acid in 100mM citric acid, 250mM Na₂HPO₄, pH4.0) was added to start color development. The reaction was stopped after 10 minutes by adding 100 μ l/well of 0.2M HCl, and shaking for 1 minute. Absorbance values at 410 nm were measured by a ELISA plate reader (Dynatec MR5000).

6.2.5. RESULTS

Results of the assay and formulae of the test substances are presented in Table III. The activity of the test substances are represented by the concentration of the test substance which produces the indicated percentage increase in the content of phosphotyrosine over the negative control. The test substances have the general formula:

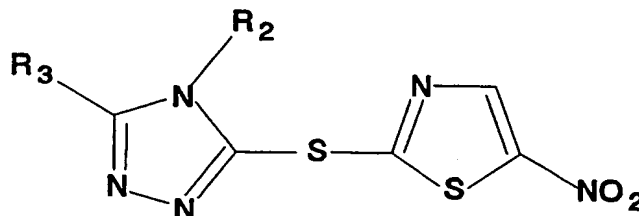


TABLE III

	R ₁	R ₂	CONCENTRATION	ACTIVITY (compared to control)
5	1-ethyl-3-methyl-pyrazole-5-yl	- (CH ₂) ₃ OCH ₃	3.9 μM	50%
	t-butyl	H	4.1 μM	50%
	thiophene-2-yl	H	77 μM	50%
10	OH	cyclohexyl	100 μM	30%
	OH	phenyl	13 μM	30%
	OH	o-trifluoromethylphenyl	24 μM	30%
	phenyl	H	53 μM	30%
	p-Chlorophenyl	H	100 μM	30%
15	OH	benzyl	200 μM 100 μM	35% 8%

Once, a test substance has been shown to be active in the assay, a range of concentrations of the compound were used in experiments wherein the target cells were incubated with the test substance for varying periods of time. The kinetics of the inhibition of dephosphorylation by the test substance at various dosage may thus be obtained.

The above results demonstrated that the assay is capable of identifying and evaluating test substances that inhibit dephosphorylation of phosphorylated tyrosine residues on the insulin receptor.

This assay may also be used for assessing any test substances for their ability to inhibit the dephosphorylation of other substrate molecules, such as insulin-like growth factor 1 receptor (IGF-1R) and epidermal growth factor receptor (EGFR). When assaying the effects of test substances on the dephosphorylation of IGF-1R, NIH3T3/IGF-1R cells expressing IGF-1R starved in serum free media were seeded in the wells of tissue culture plates at a density 20,000 cells/well. The wells of ELISA plate were coated with anti-IGF-1R antibodies. For assaying the effects on EGFR

dephosphorylation, NIH3T3/EGFR cells expressing EGFR grown in media containing 0.5% for 40 hours were seeded in the wells of 96-well tissue culture plates at a density 10,000 cells/well. The wells of ELISA plate were coated with anti-EGFR antibodies.

7. EXAMPLE: CELL-FREE SCREENING ASSAY FOR KINASE ACTIVITY

This assay provides a method for measuring the effects of test substances on the kinase activity of specific targets in a cell-free system. The target substrate is isolated from cell lysates by immobilization in microtiter plate wells coated with anti-substrate antibody. Following addition of the test substance, ATP and a bivalent cation are added to the wells to initiate the kinase reaction. Concentrations of ATP can be varied to assess whether a test compound competes for ATP or substrate interaction. The concentration of ATP should be < 3 μ M for assessing high sensitivity in the kinase reaction. A concentration of 10 - 50 μ M ATP, is preferred for assessing inhibition of ATP interaction, and most preferably 50 μ M. At this ATP concentration the assay will preferentially identify inhibitors that do not compete well with ATP. Phosphorylation of tyrosine residues on the immobilized target is then detected using antiphosphotyrosine antiserum.

7.1. MATERIALS AND REAGENTS

The materials and reagents used are described in Section 6.1 with the exception that the cells used to prepare the cell lysate were another EGFR over-expressing human epidermoid carcinoma cell line, A431 (ATCC CRL 1555). Test substances were obtained from LC Service Corp (Woburn, MA).

7.2. PROCEDURE

7.2.1. PREPARATION OF ASSAY PLATES

Preparation of the assay plates was performed as
5 described in Section 6.2.1.

7.2.2. PREPARATION OF CELL LYSATE

Cells (A431) were grown to 80-90% confluency in DMEM
(Gibco) plus 10% calf serum then washed twice with PBS. HNTG
10 was added and the dish place on ice for five minutes. Cells
were scraped from the plate and homogenized as described in
Section 6.2.3, transferred to a test tube, and cleared of cell
debris by centrifugation (10,000 x g, 10 minutes, 4°C). The
cleared lysate can be stored at -80 C until used.

15

7.2.3. ASSAY PROCEDURE

Seven μg of cell lysate was added to each well of a
prepared assay plate plus PBS to make a final volume of 150
 μl . The plate was incubated for 30 minutes, shaking, at room
20 temperature, then washed five times with TBST. Serial
dilutions of test compound stocks (10 mg/ml in DMSO) were
diluted 1:20 into 5% DMSO in PBS for a final concentration
range of 150 μM to 1 nM, then 135 μl was added per well. The
plate was incubated for 30 minutes, shaking, at room
25 temperature.

The kinase reaction was initiated by the addition of 15
 μl of ATP/Mn mix (in 50 mM MnCl_2 , final concentration of ATP 1
 μM , 3 μM or 10 μM) for a total volume of 150 μl . The plate
was incubated for 5 minutes at room temperature, shaking, then
30 washed 5 times with TBST. The amount of phosphorylation
present was measured as described in Section 6.2.3.

35

7.3. RESULTS

TABLE IV
EFFECT OF TEST SUBSTANCES ON
EGF-R KINASE ACTIVITY A CELL FREE SYSTEM

5	Test	Substance	ATP Conc. (μ m)	IC50 (μ M)
		Genestein	10	150
			3	10
10			1	6
		Lavendustin A	10	55
			3	0.1
			1	0.08
15		Staurosporin	10	100
			3	10
			1	8
		Herbimycin A	1	>100

8. EXAMPLE: CELL FREE PHOSPHATASE ASSAY

20 This assay provides a method for measuring the effects of test substances on the phosphatase activity of PTP 1B as measured by dephosphorylation of EGFR. The assay protocol used is substantially the same as that described in Section 7.

25 8.1. MATERIALS AND REAGENTS

The materials and reagents used are substantially the same as those described in Section 7.1 with the following additions.

- 30 1. TBS: 50 mM Tris, pH 7.2, 150 mM NaCl
2. PTP 1B: the protein was produced in bacterial cells (strain B121) using a pET plasmid with a T7 promoter, Tonks, et al. Methods in Enzymology 201:427-443, 1991.
- 35 3. phosphatase buffer: 100 mM Tris, pH 7.2, 5% DMSO, 1.5 mM NaPyrophosphate.

8.2. PROCEDURE

8.2.1. PREPARATION OF ASSAY PLATES

Preparation of the assay plates was performed as
5 described in Section 7.2.1.

8.2.2. PREPARATION OF CELL LYSATE

Preparation of the cell lysate was performed as described
in Section 7.2.1.

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8.2.3. ASSAY PROCEDURE

The assay procedure used was substantially the same as
that described in Section 8.2.3 with the following changes.
After binding of the EGFR substrate to the microtiter wells
15 and washing 5 times with distilled water and once with TBST, a
kinase reaction was initiated by adding 135 μ l of TBS per
well, then 15 μ l of 0.003 mM ATP in 50 mM MnCl₂, for a final
concentration of 3 μ M ATP and 5mM MnCl₂. The reaction was
allowed to proceed for 5 minutes, shaking, then stopped with
20 the addition of 16.5 μ l of 200 mM EDTA, pH 8.0, shaking
continuously during this addition. The plate was left shaking
for an additional minute then washed 5 times with distilled
water and once with TBST.

Test compounds were diluted 1:100 in phosphatase buffer,
25 and aliquots of 140 μ l were added to each well. Control wells
received phosphatase buffer alone.

The phosphatase reaction was initiated by the addition of
PTP 1B diluted in 100 mM Tris, pH 7.2 (10 μ l (20 ng) per
well). The plate was incubated for 15 minutes, shaking, then
30 washed 5 times with distilled water and once with TBST. The
amount of phosphotyrosine remaining is measured as described
in Section 6.2.3.

8.3. RESULTS

35 The results shown in Table V below demonstrate that test
compounds can be assessed quantitatively for their ability to
inhibit phosphatase activity in a cell free system.

TABLE V
EFFECT OF TEST SUBSTANCES ON PHOSPHATASE 1B
ACTIVITY ON THE EGFR IN VITRO

5	Test	
	Substance	IC50 (μ M)
	NaVanadate	30

10 The present invention is not to be limited in scope by
the specific embodiments described which are intended as
single illustrations of individual aspects of the invention,
and functionally equivalent methods and components are within
the scope of the invention. Indeed, various modifications of
the invention, in addition to those shown and described herein
15 will become apparent to those skilled in the art from the
foregoing description and accompanying drawings. Such
modifications are intended to fall within the scope of the
appended claims.

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WHAT IS CLAIMED IS:

1. An assay for screening test substances for the ability to modulate activity of a tyrosine phosphatase involved in signal transduction, comprising:
- 5 (a) contacting a lysate of a target cell, after the target cell was exposed to a test substance, with an anchoring molecule specific for a protein substrate which is dephosphorylated as a result of signal transduction in the target cell, under conditions and for a time sufficient to allow binding of the protein substrate to the anchoring molecule; and
- 10 (b) detecting phosphotyrosine residues on any protein substrate bound to the anchoring molecule, in which differences in the detection of phosphotyrosine residues on the immobilized protein substrate derived from lysates of the target cells which were exposed to the test substance as compared to that of immobilized protein substrate derived from control target cells which were not exposed to the test substance, indicate that the test substance modulates the activity of the tyrosine phosphatase.
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2. The assay of Claim 1 in which the anchoring molecule is an antibody.

- 30 3. The assay of Claim 1 in which the phosphotyrosine residue is detected using an antibody specific for phosphotyrosine.

4. The assay of Claim 3 in which the antibody specific for phosphotyrosine is a monoclonal antibody.

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5. The assay of Claim 3 in which the antibody specific for phosphotyrosine is labeled.

6. The assay of Claim 5 in which the label is selected from the group consisting of a radiolabel, a fluorescent label, a luminescent label and an enzymatic label.

7. The assay of Claim 3 in which the antibody specific for phosphotyrosine is indirectly labeled.

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8. The assay of Claim 7 in which the antibody specific for phosphotyrosine is indirectly labeled with a label selected from the group consisting of a radiolabeled anti-immunoglobulin, a fluorescently labeled anti-immunoglobulin, a luminescently labeled anti-immunoglobulin, and an enzymatically labeled anti-immunoglobulin.

9. The assay of Claim 2 in which the antibody is bound to a microtiter plate.

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10. An assay for screening test substances for the ability to modulate activity of a tyrosine kinase involved in signal transduction, comprising:

- 25 (a) reacting the test substance with a protein substrate that is phosphorylated in a reaction mixture containing the tyrosine kinase and ATP under conditions and for a time sufficient to allow phosphorylation of the protein substrate, and
- 30 (b) detecting phosphotyrosine residues on the protein substrate, in which differences in the detection of phosphotyrosine residues on the protein substrate in the reaction mixture as compared to a control reaction mixture that
- 35 does not contain the test substance indicate that the test substance modulates the activity

of the tyrosine kinase involved in signal transduction.

11. The assay of Claim 10 in which the protein substrate
5 is immobilized by an antibody specific for the protein substrate bound to a solid surface.

12. The assay of Claim 11 in which the immobilized antibody is a monoclonal antibody.

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13. The assay of Claim 10 in which the protein substrate is immobilized prior to conducting the reaction.

14. The assay of Claim 10 in which the protein substrate
15 is immobilized subsequent to conducting the reaction.

15. The assay of Claim 11 in which the solid surface is a microtiter well plate.

20 16. The assay of Claim 10 in which phosphotyrosine residues are detected using an antibody specific for phosphotyrosine.

17. The assay of Claim 11 in which the antibody specific
25 for phosphotyrosine is a monoclonal antibody.

18. The assay of Claim 11 in which the antibody specific for phosphotyrosine is labeled.

30 19. The assay of Claim 18 in which the label is selected from the group consisting of a radiolabel, a fluorescent label, a luminescent label and an enzymatic label.

20. The assay of Claim 18 in which the antibody is
35 indirectly labeled.

21. The assay of Claim 20 in which the antibody is indirectly labeled with a label selected from the group consisting of a radiolabeled anti-immunoglobulin, a fluorescently labeled anti-immunoglobulin, a luminescently
5 labeled anti-immunoglobulin and an enzymatically labeled anti-immunoglobulin.

22. A kit comprising:

- 10 (a) a solid phase coated with an anchoring molecule to a substrate of interest; and
(b) a detection molecule that can detect the phosphorylation or dephosphorylation of the substrate of interest.

15 23. The kit of Claim 22 further comprising a protein kinase or protein phosphatase.

24. An assay for screening test substances for the ability to modulate activity of a tyrosine kinase involved in
20 signal transduction, comprising:

- 25 (a) contacting a lysate of a target cell, after the target cell was exposed to a test substance, with an immobilized antibody specific for a protein substrate which is phosphorylated as a result of signal transduction in the target cell, under conditions and for a time sufficient to allow binding of the protein substrate to the anchoring molecule; and
30 (b) detecting phosphotyrosine residues on any protein substrate bound to the immobilized antibody, in which differences in the detection of phosphotyrosine residues on the immobilized protein substrate derived from lysates of the target cells which were exposed to the test
35 substance as compared to that of immobilized protein substrate derived from control target cells which were not exposed to the test

substance, indicate that the test substance modulates the activity of the tyrosine kinase.

25. An assay for screening test substances for the ability to modulate the level of phosphotyrosine on a protein substrate involved in signal transduction, comprising:

- 10 (a) contacting a lysate of a target cell, after the target cell was exposed to a test substance, with an immobilized antibody specific for a protein substrate which is phosphorylated or dephosphorylated as a result of signal transduction in the target cell, under conditions and for a time sufficient to allow binding of the protein substrate to the immobilized antibody; and
- 15 (b) detecting phosphotyrosine residues on any protein substrate bound to the immobilized antibody, in which differences in the detection of phosphotyrosine residues on the immobilized protein substrate derived from lysates of the target cells which were exposed to the test substance as compared to that of immobilized protein substrate derived from control target cells which were not exposed to the test substance, indicate that the test substance modulates the phosphotyrosine level of the protein substrate.

26. The assay of Claim 1, 24 or 25 wherein the test substance is a therapeutic compound that modulates kinase or phosphatase activity and the assay is used to determine whether said target cell will respond to said therapeutic compound.

27. The assay of Claim 26, in which said target cell is derived from a patient exhibiting a disease state that is related to the dysfunction of said signal transduction in said

target cell, thereby determining whether said target cell will respond to said therapeutic compound.

28. The assay of Claim 27, in which the disease state is
5 selected from the group consisting of neoplasia, cancer,
diabetes, anemia, immunodeficiency, inflammation, rheumatoid
arthritis, psoriasis, neurodegenerative diseases, bone
diseases, osteoporosis and metabolic disorders.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08332**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9.2; 435/7.1, 7.4, 7.6, 7.71, 7.72, 7.91, 17, 21, 174; 436/518, 524, 535, 548

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, MEDLINE

search terms: screening assays, tyrosine phosphatase, tyrosine kinase, immunoassay, inhibitors, activators, modulate activity

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CLEAVELAND et al. A Microtiter-Based Assay for the Detection of Protein Tyrosine Kinase Activity. Analytical Biochemistry. 1990, Vol. 190, pages 249-253, see entire document.	1-26
X	KING et al. High Throughput Assay for Inhibitors of the Epidermal Growth Factor Receptor-Associated Tyrosine Kinase. Life Sciences. 1993, Vol. 53, pages 1465-1472, see entire document.	24, 26
----		-----
Y		27, 28



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*&* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

08 AUGUST 1996

Date of mailing of the international search report

04 SEP 1996

Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/08332

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FARLEY et al. Development of Solid-Phase Enzyme-Linked Immunosorbent Assays for the Determination of Epidermal Growth Factor Receptor and pp60 Tyrosine Protein Kinase Activity. Analytical Biochemistry. 1992, Vol. 203, pages 151-157, see entire document.	1-28
A	RIJKSEN et al. A Nonradioactive Dot-Blot Assay for Protein Tyrosine Kinase Activity. Analytical Biochemistry. 1989, Vol. 182, pages 98-102, see entire document.	1-28
A	WO 90/10234 A1 (G. RIJKSEN) 07 September 1990 (07/09/90), see entire document.	1-28

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08332

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐
☒

The additional search fees were accompanied by the applicant's protest.
No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08332

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 49/00; G01N 31/00, 33/48, 33/53, 33/573, 33/542, 33/543, 33/551, 33/544; C12Q 1/50, 1/42, 1/00, 1/16

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/9.2; 435/7.1, 7.4, 7.6, 7.71, 7.72, 7.91, 17, 21, 174; 436/518, 524, 535, 548

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-9 and 26-28, drawn to an assay for screening test substances for the ability to modulate activity of a tyrosine phosphatase.

Group II, claims 10-21, 24 and 26-28, drawn to an assay for screening test substances for the ability to modulate activity of a tyrosine kinase.

Group III, claims 22-23, drawn to a kit.

Group IV, claims 25-28, drawn to an assay for screening test substances for the ability to modulate the level of phosphotyrosine on proteins.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: no single method or pathway is recited to detect the level of phosphotyrosine residues on protein substrate. The assays are directed toward the detection of the ability of test substances to modulate activity of different enzymes. The step of detecting phosphoryrosine is not considered a technical feature because phosphotyrosine can be detected using multiple assays based on multiple pathways of production, such as via tyrosine kinase phosphorylation and/or generic protein tyrosine or protein phosphatase.

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